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miR-342-3p suppresses proliferation, migration and invasion by targeting FOXM1 in human cervical cancer

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ABSTRACT

FOXM1 is a well-established oncogenic factor that has been reported to be involved in multiple biological processes including cell proliferation, growth, angiogenesis, migration and invasion. It can also be regulated by miRNAs. In this study, we reported that FOXM1 is directly targeted by miR-342-3p, which is down-regulated along with its host gene, EVL, in human cervical cancer tissues compared to the adjacent normal tissues. Functional studies suggested that the overexpression of miR-342-3p inhibits cell proliferation, migration and invasion in cervical cell lines. FOXM1 is upregulated and negatively correlates with miR-342-3p in cervical cancer tissues, and the overexpression of FOXM1 rescues the phenotype changes induced by the overexpression of miR-342-3p.

1. Introduction

Mammalian transcription factor Forkhead Box M1 (FOXM1) belongs to the extensive family of Forkhead transcription factors, whose members harbor 100 amino acids and an evolutionarily conserved DNA binding domain called Forkhead or winged-helix domain [1,2]. Genome-wide gene expression profiles of cancer have shown that FOXM1 is commonly upregulated in a diverse array of human solid tumors, including liver, lung, colon, pancreatic, brain, breast, genital system (prostate, cervical and ovarian tumors), as well as hematological and nervous system tumors [3,4]. It is demonstrated that FOXM1 is involved in cell proliferation, cell cycle progression, apoptosis, cell differentiation, and angiogenesis, and plays an essential role in the invasion, metastasis, and epithelial–mesenchymal transitions in cancer [5]. Moreover, FOXM1 has been reported to be regulated by miRNAs, for example, miR-134 in lung cancer [6] and miR-370 in acute myeloid leukemia [7].

MicroRNAs (miRNAs) are ubiquitous, ~22-nucleotide (nt), non-coding regulators that modulate essential cellular processes at the post-transcriptional level [8]. Accumulating evidence suggests that aberrantly expressed miRNAs lead to various diseases, including cancer [9]. Among them, cervical cancer is a primary cancer in females with a high incidence all over the world [10]. It is reported that a number of miRNAs are aberrantly expressed in cervical cancer. miR-21 is overexpressed in cervical cancer and is a negative regulator of expression of the tumor suppressor gene programmed cell death 4 (PDCD4) [11]. miR-19a and miR-19b are overexpressed in cervical cancer cells and enhance cell growth and invasion by targeting Cullin 5 (CUL5) [12]. Both miRNAs act as onco-miRNAs. However, some tumor suppressor miRNAs were also studied in cervical cancer. For example, miR-506 exerts its anti-proliferative function by directly targeting Gli3 and acts as a tumor suppressor by inhibiting cervical cancer growth in vitro and in vivo [13]. Expression of hsa-miR-342, a microRNA encoded in an intron of the gene EVL, is commonly suppressed in human colorectal cancer (CRC) [14]. miR-342 is involved in the regulation of DNA methyltransferase 1 (DNMT1) to inhibit CRC cell proliferation and metastasis both in vitro and in vivo [15].

A large cohort of studies provided evidence for the pivotal role of FOXM1 in the regulation of cell proliferation, migration, and invasion, as well as metastasis in various cancers. However, evidence of a biological association between FOXM1 and miR-342-3p in cervical cancer has not been reported. In the present study, we found that FOXM1 was directly targeted by miR-342-3p. In

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coordination with its host gene EVL, miR-342-3p was downregulated in cervical intraepithelial neoplasia (CIN) tissues, especially in cervical cancer tissues. Moreover, the overexpression of miR-342-3p mimics suppressed cell growth in vitro and in vivo and also inhibits the migration and invasion of the HeLa and C33A cell lines. Restoration of FOXM1 abolished the suppression of proliferation, migration and invasion induced by miR-342-3p mimics in cervical cancer cell lines.

2. Materials and methods

2.1. Cell culture and transfection

The human cervical cancer cell lines are maintained in the following medium: HeLa and Caski in RPMI1640 (GIBCO, USA), and C33A and Siha in Eagle's Minimum Essential Medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 μg/ml of streptomycin. The cell lines were incubated at 37 °C in a humidified chamber supplemented with 5% CO_2. Transfection was performed with Lipofectamine 2000 Reagent (Invitrogen) following the manufacturer's protocol.

2.2. Clinical tumor specimens

Twenty-seven pairs of cervical cancer samples and matched normal cervical tissues as well as CIN tissues (CIN I: 35 samples; CIN II: 35 samples; and CIN III: 45 samples) were collected from 2012 to 2014 in Qingdao Haici Hospital. Among them, pathological analysis confirmed that the cancer tissues are human cervical squamous cancer (low-differentiation: 9 samples; mid-differentiation: 13 samples; and high-differentiation: 5 samples). All of the samples were obtained with the patients' informed consent and approved by the Ethics Committee of Qingdao Haici Hospital. The patient clinical information is shown in Table 1. The category of cervical samples was confirmed by pathological analysis. The RNA from tissue samples was isolated using a miRVana™ miRNA Isolation Kit (Ambion) following the manufacturer's instructions.

2.3. Luciferase reporter assay

For luciferase reporter experiments, HeLa and C33A cells were seeded at 1.5×10^5 cells per well in a 24-well plate and were co-transfected 24 h later with pGL3-control (400 ng), wild-type pGL3-FOXM1 (400 ng), mutant pGL3-FOXM1 (400 ng), pGL4.73 vector (50 ng; Promega) and miR-342-3p mimics (50 nM) or negative control miRNA (scramble, 50 nM) using Lipofectamine 2000 (Invitrogen). The cells were harvested 48 h after transfection, and firefly and Renilla luciferase activities were analyzed with the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega).

2.4. Western blot and immunohistochemistry assays

Total cellular proteins were extracted and separated by SDS–PAGE gels, and Western blot analysis was performed according to standard procedures. β-Actin on the same membrane was used as a loading control. The primary antibodies included mono-clonal anti-FOXM1 (1:2000, Abcam) and anti-GAPDH (1:5000, Abcam). Proteins were visualized using the ECL procedure (Amersham Biosciences, Sweden). The bands of gray intensity were analyzed by Image J. IHC was performed according to the methods described previously (1). After pretreatment with a microwave, the sections were blocked and incubated using monoclonal mouse anti-FOXM1 (Abcam). The staining intensity was assessed.

Table 1
Patient clinical information.

Index	Gender	Age	Clinical diagnosis
1	Female	50	CIN I
2	Female	46	CIN I
3	Female	43	CIN I
4	Female	44	CIN I
5	Female	28	CIN I
6	Female	41	CIN I
7	Female	33	CIN I
8	Female	41	CIN I
9	Female	27	CIN I
10	Female	48	CIN I
11	Female	52	CIN I
12	Female	40	CIN I
13	Female	35	CIN I
14	Female	29	CIN I
15	Female	56	CIN I
16	Female	50	CIN I
17	Female	47	CIN I
18	Female	42	CIN I
19	Female	65	CIN I
20	Female	46	CIN I
21	Female	52	CIN I
22	Female	47	CIN I
23	Female	41	CIN I
24	Female	28	CIN I
25	Female	36	CIN I
26	Female	40	CIN I
27	Female	42	CIN I
28	Female	45	CIN I
29	Female	49	CIN I
30	Female	54	CIN I
31	Female	36	CIN I
32	Female	35	CIN I
33	Female	30	CIN I
34	Female	54	CIN I
35	Female	50	CIN I
36	Female	68	CIN II
37	Female	32	CIN II
38	Female	35	CIN II
39	Female	34	CIN II
40	Female	29	CIN II
41	Female	41	CIN II
42	Female	43	CIN II
43	Female	47	CIN II
44	Female	37	CIN II
45	Female	43	CIN II
46	Female	40	CIN II
47	Female	39	CIN II
48	Female	39	CIN II
49	Female	33	CIN II
50	Female	36	CIN II
51	Female	24	CIN II
52	Female	34	CIN II
53	Female	41	CIN II
54	Female	40	CIN II
55	Female	35	CIN II
56	Female	33	CIN II
57	Female	28	CIN II
58	Female	42	CIN II
59	Female	46	CIN II
60	Female	39	CIN II
61	Female	30	CIN II
62	Female	41	CIN II
63	Female	55	CIN II
64	Female	45	CIN II
65	Female	41	CIN II
66	Female	36	CIN II
67	Female	33	CIN II
68	Female	38	CIN II
69	Female	23	CIN II
70	Female	29	CIN II
71	Female	35	CIN III
72	Female	23	CIN III
73	Female	44	CIN III
74	Female	45	CIN III

(*continued on next page*)

Table 1 (*continued*)

Index	Gender	Age	Clinical diagnosis
75	Female	44	CIN III
76	Female	38	CIN III
77	Female	46	CIN III
78	Female	61	CIN III
79	Female	39	CIN III
80	Female	32	CIN III
81	Female	46	CIN III
82	Female	33	CIN III
83	Female	48	CIN III
84	Female	55	CIN III
85	Female	37	CIN III
86	Female	27	CIN III
87	Female	29	CIN III
88	Female	40	CIN III
89	Female	46	CIN III
90	Female	40	CIN III
91	Female	37	CIN III
92	Female	40	CIN III
93	Female	45	CIN III
94	Female	50	CIN III
95	Female	25	CIN III
96	Female	39	CIN III
97	Female	37	CIN III
98	Female	32	CIN III
99	Female	43	CIN III
100	Female	47	CIN III
101	Female	47	CIN III
102	Female	38	CIN III
103	Female	37	CIN III
104	Female	47	CIN III
105	Female	38	CIN III
106	Female	40	CIN III
107	Female	50	CIN III
108	Female	53	CIN III
109	Female	38	CIN III
110	Female	36	CIN III
111	Female	31	CIN III
112	Female	69	CIN III
113	Female	64	CIN III
114	Female	42	CIN III
115	Female	50	CIN III
116	Female	38	Cervical squamous cancer
117	Female	47	Cervical squamous cancer
118	Female	48	Cervical squamous cancer
119	Female	62	Cervical squamous cancer
120	Female	34	Cervical squamous cancer
121	Female	46	Cervical squamous cancer
122	Female	40	Cervical squamous cancer
123	Female	69	Cervical squamous cancer
124	Female	49	Cervical squamous cancer
125	Female	38	Cervical squamous cancer
126	Female	42	Cervical squamous cancer
127	Female	34	Cervical squamous cancer
128	Female	41	Cervical squamous cancer
129	Female	37	Cervical squamous cancer
130	Female	55	Cervical squamous cancer
131	Female	50	Cervical squamous cancer
132	Female	43	Cervical squamous cancer
133	Female	66	Cervical squamous cancer
134	Female	41	Cervical squamous cancer
135	Female	46	Cervical squamous cancer
136	Female	46	Cervical squamous cancer
137	Female	52	Cervical squamous cancer
138	Female	63	Cervical squamous cancer
139	Female	48	Cervical squamous cancer
140	Female	43	Cervical squamous cancer
141	Female	68	Cervical squamous cancer
142	Female	58	Cervical squamous cancer

2.5. MTT and colony formation assays

The 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay was used to evaluate cell viability and proliferation. The spectrophotometric absorbance of each sample was measured at 570 nm. All experiments were performed in triplicate, and the average results were calculated. For the colony formation assay, tumor cells (200 cells per well in a 12-well plate) overexpressing miR-342-3p mimics or scramble were seeded in complete medium. The cells were grown for 10 days at 37 °C with 5% CO_2. Colony formation was visualized with crystal violet staining. The number of colonies with more than 50 cells were counted.

2.6. Migration and invasion assays

For the transwell migration assays, 6×10^4 HeLa cells and 10×10^4 C33A cells were plated in the top chamber with a non-coated membrane (24-well insert; 8-μm pore size; BD Biosciences). For the invasion assays, 1×10^5 HeLa cells and 2×10^5 C33A cells were plated in the top chamber with a Matrigel-coated membrane (24-well insert; 8-μm pore size; BD Biosciences). For both assays, the cells were plated in serum-free medium, and medium supplemented with 20% serum (GIBCO) was used as a chemoattractant in the lower chamber. The cells were incubated for 16 h at 37 °C and 5% CO_2 in a tissue culture incubator. After 16 h, the non-migrated/non-invaded cells were removed from the upper sides of the transwell membrane filter inserts using cotton-tipped swabs. The migrated/invaded cells on the lower sides of the inserts were stained with crystal violet, and five random fields of the cells were counted.

2.7. Xenograft experiments

Equal numbers of HeLa cells (10^6) with forced expression of miR-342-3p mimics or scramble were suspended in 100 μl PBS and injected subcutaneously into the right rear flank of each mouse (5 mice per group). Tumor growth was monitored every 2 days in each group. The tumor volume was calculated using the formula $V = 1/2\ a \times b2$, where a is the longest tumor axis and b is the shortest tumor axis. The mice were sacrificed 20 days later.

2.8. Statistical analysis

SPSS 17.0 software was used for the statistical analysis. The data are presented as the mean and standard deviation (s.d.). Group comparisons were performed using Student's t-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. miR-342-3p is down-regulated in cervical cancer tissue and cell lines

To elucidate the role of miR-342-3p in human cervical cancer development, we detected expression levels of miR-342-3p and its host gene, EVL, in 27 cervical cancer tissue samples and paired adjacent normal tissue samples by qRT-PCR. As shown in Fig. 1A and B, the expression levels of the EVL mRNA and miR-342-3p were generally reduced in cervical cancer tissues compared to those in adjacent normal tissues. To further study whether the downregulation of miR-342-3p and its host gene, EVL mRNA, is specific to cervical cancer, we used qRT-PCR to analyze their expression levels in cervical intraepithelial neoplasia (CIN) samples (CIN I: 35 samples; CIN II: 35 samples; and CIN III: 45 samples) and cervical cancer samples. The data showed that the expression levels of EVL mRNA and miR-342-3p were both relatively reduced in the CIN tissues, irrespective of the grade, but the levels were much lower in cervical cancer tissues than those in the CIN tissues (Fig. 1C and D). Subsequently, we assessed the expression levels

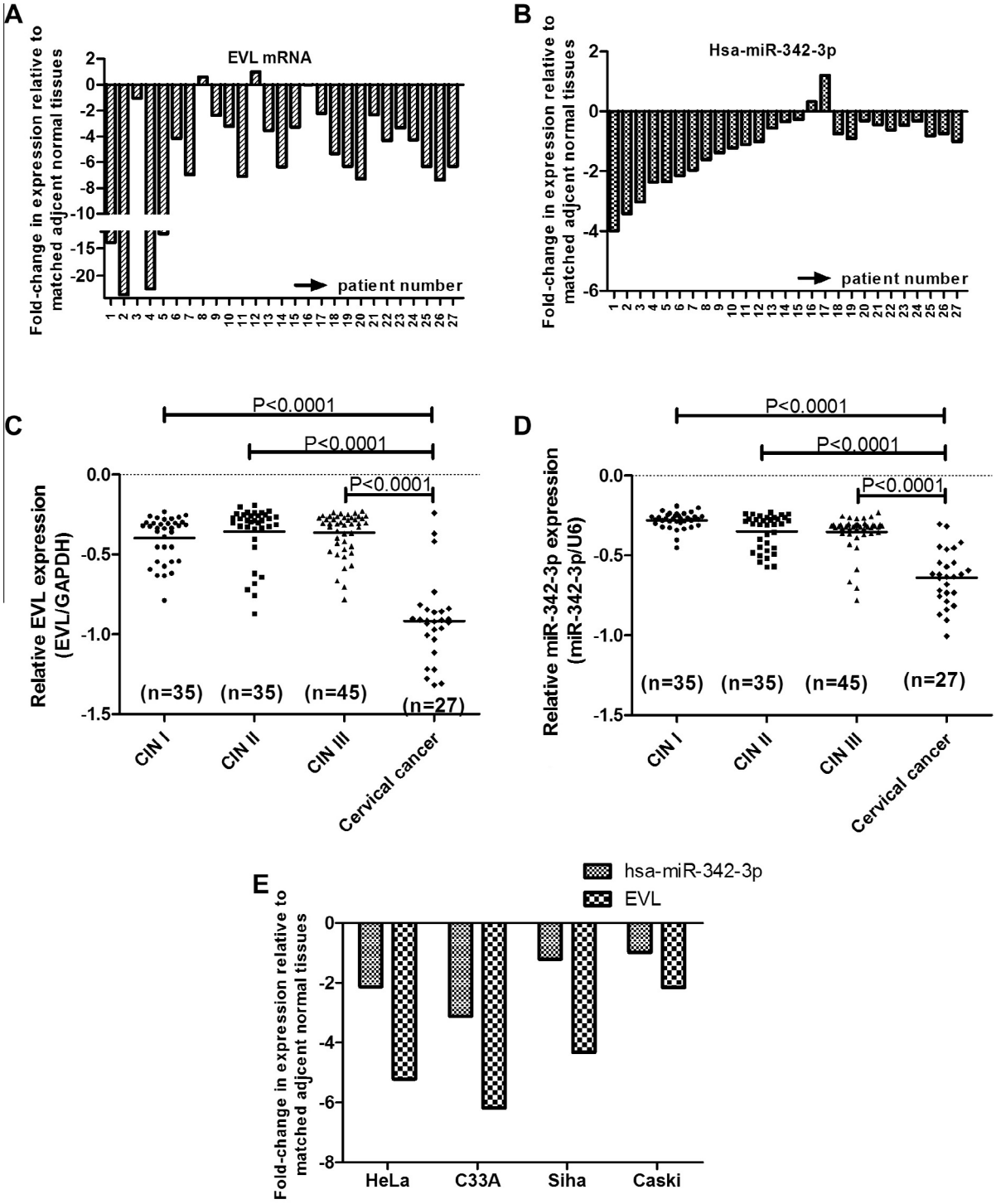

Fig. 1. miR-342-3p and its host gene, EVL, are generally down-regulated in human cervical cancer tissues and cell lines. The expression levels of miR-342-3p (B) and its host gene EVL (A) were analyzed by qRT-PCR with human CIN samples (CIN I: 35 samples; CIN II: 35 samples; and CIN III: 45 samples) and cervical cancer tissues (27 samples). The levels of miR-342-3p and EVL mRNA were lower in cervical cancer tissues than in CIN tissues, although their levels in CIN tissues (grade I–III) were relatively low (C and D). The expression of two RNAs was relatively down-regulated in the HeLa, C33A, Siha and Caski cell lines by qRT-PCR (E). $^{*}P < 0.05$. The experiments were performed in triplicate.

of EVL mRNA and miR-342-3p by qRT-PCR in four cervical cancer cell lines: HeLa, C33A, Siha and Caski. We used the relative expression levels of EVL mRNA and miR-342-3p of the respective loading controls in the four cell lines due to the lack of a normal cervical cell line. Although the degree of downregulation varied from one cell line to another, their expression was relatively low overall. As the potential of downregulation is greater in the HeLa and C33A cell lines than that in the Siha and Caski cell lines (Fig. 1E), further studies were performed in the HeLa and C33A cell lines. These data indicate that miR-342-3p and its host gene, EVL mRNA, are down-regulated in CIN and cervical cancer tissues, as well as in cervical cell lines.

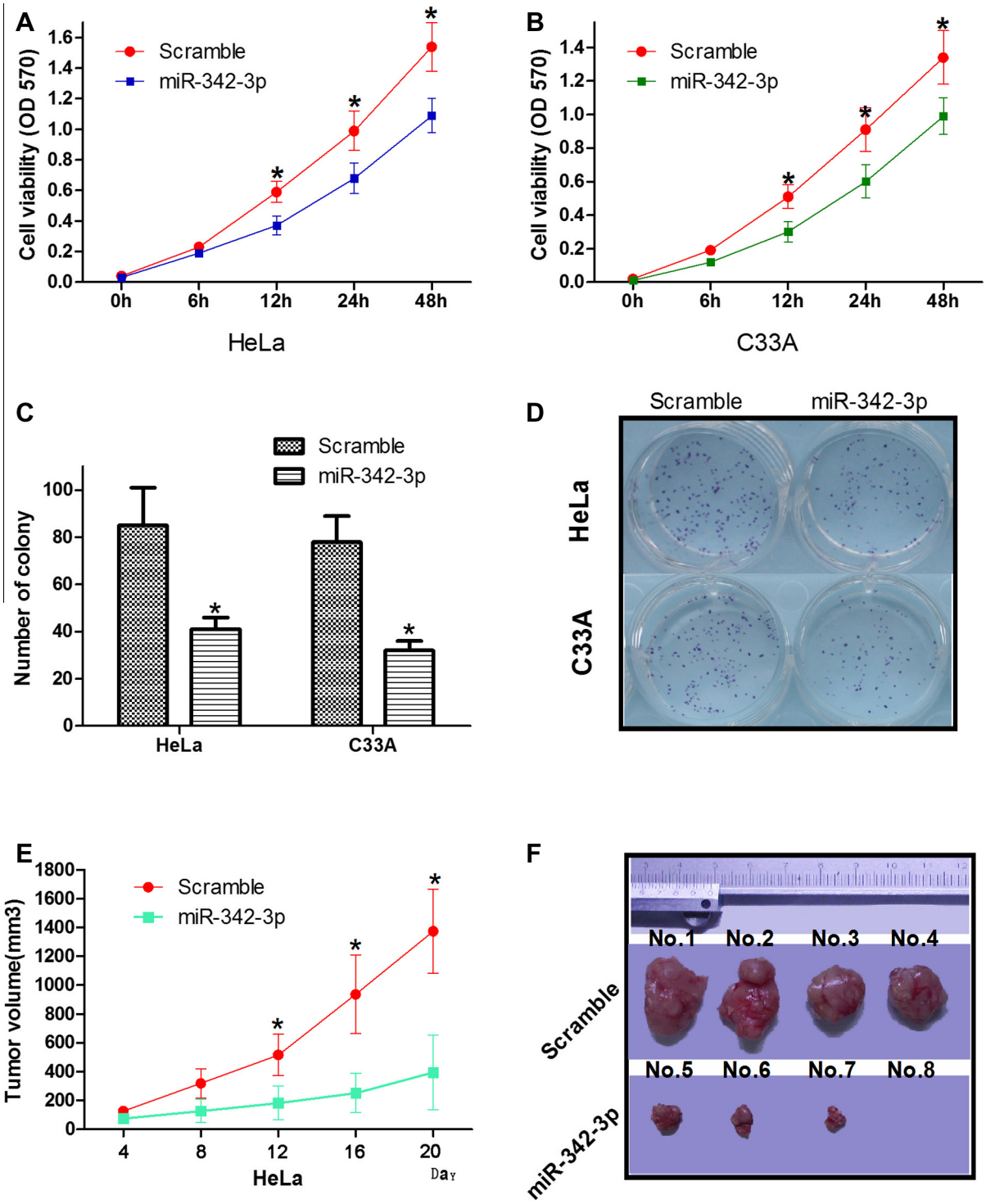

Fig. 2. miR-342-3p directly targets FOXM1. (A) The miR-342-3p binding sites located on the FOXM1 mRNA 3′UTR. (B) miR-342-3p or scramble and wild-type or mutant FOXM1 3′UTR were co-transfected into HeLa cells. miR-342-3p reduced the luciferase activities with wild-type FOXM1 3′UTR and did not alter the luciferase activities with mutant FOXM1 3′UTR. (C) miR-342-3p decreased endogenous FOXM1 mRNA expression in HeLa and C33A cells. (D) Western blot assays showed that the expression of FOXM1 protein was up-regulated in HeLa and C33A cells and down-regulated in Siha and Caski cells. (E) Western blot was used to detect the expression of FOXM1 protein in HeLa cells by ectopic expression of miR-342-3p and in Siha cells by blocking miR-342-3p. GAPDH is a loading control. $^{*}P < 0.05$. The experiments were performed in triplicate.

3.2. FOXM1 is a direct target of miR-342-3p

FOXM1 has been reported to be an important molecule that triggers metastasis and invasion in various cancers. Using prediction tools, we predicted that the target miRNA of FOXM1 was miR-342-3p. To further confirm that miR-342-3p directly targets oncogene FOXM1, we performed luciferase reporter assays to examine whether miR-342-3p interacts directly with its target oncogenic FOXM1. We identified a putative binding site of miR-342-3p in the FOXM1 mRNA 3′UTR (Fig. 2A). The fragments were then cloned downstream of the luciferase gene with a stop codon in the pGL3-control vector. Subsequently, after validation of the miR-342-3p mimics in the HeLa and C33A cell lines (Fig. 4A and B), we performed the luciferase assay in HeLa cells. As shown in

Fig. 2B, the reporter with the wild-type 3′UTR of FOXM1 showed markedly lower luciferase activity in HeLa cells expressing miR-342-3p mimics compared with those expressing negative control miRNA; however, the reduced luciferase activity caused by the wild-type FOXM1 mRNA 3′UTR was abolished by mutant 3′UTR. To further validate the association between miR-342-3p and FOXM1, we detected endogenous FOXM1 mRNA in the HeLa and C33A cell lines transfected with miR-342-3p mimics or a non-specific miRNA (as a negative control). qRT-PCR showed that miR-342-3p mimics dramatically decreased the expression of endogenous FOXM1 mRNA (Fig. 2C). Next, western blots analyzed the protein level of FOXM1 in the four cervical lines: HeLa, C33A, Siha, and Caski. As shown in Fig. 2D, the protein levels in the HeLa and C33A lines were higher than those in the Siha and Caski lines. Thus, HeLa cells transfected with miR-342-3p mimics and Siha cells transfected with anti-sense oligomer of miR-342-3p (anti-miR-342-3p) were adopted. Western blots showed that the expression of FOXM1 protein was decreased by approximately 70% in HeLa cells transfected with miR-342-3p mimics and that the FOXM1 protein level was higher in Siha cells transfected with anti-miR-342-3p compared with the control group cells. Taken together, these results suggest that FOXM1 is a direct target gene of miR-342-3p.

3.3. miR-342-3p suppresses proliferation, growth, invasion and migration of human cervical cells

To investigate the roles of miR-342-3p in cervical cancer cells, MTT assays and colony formation assays were used to evaluate whether miR-342-3p affects cell viability and growth. Overexpres-

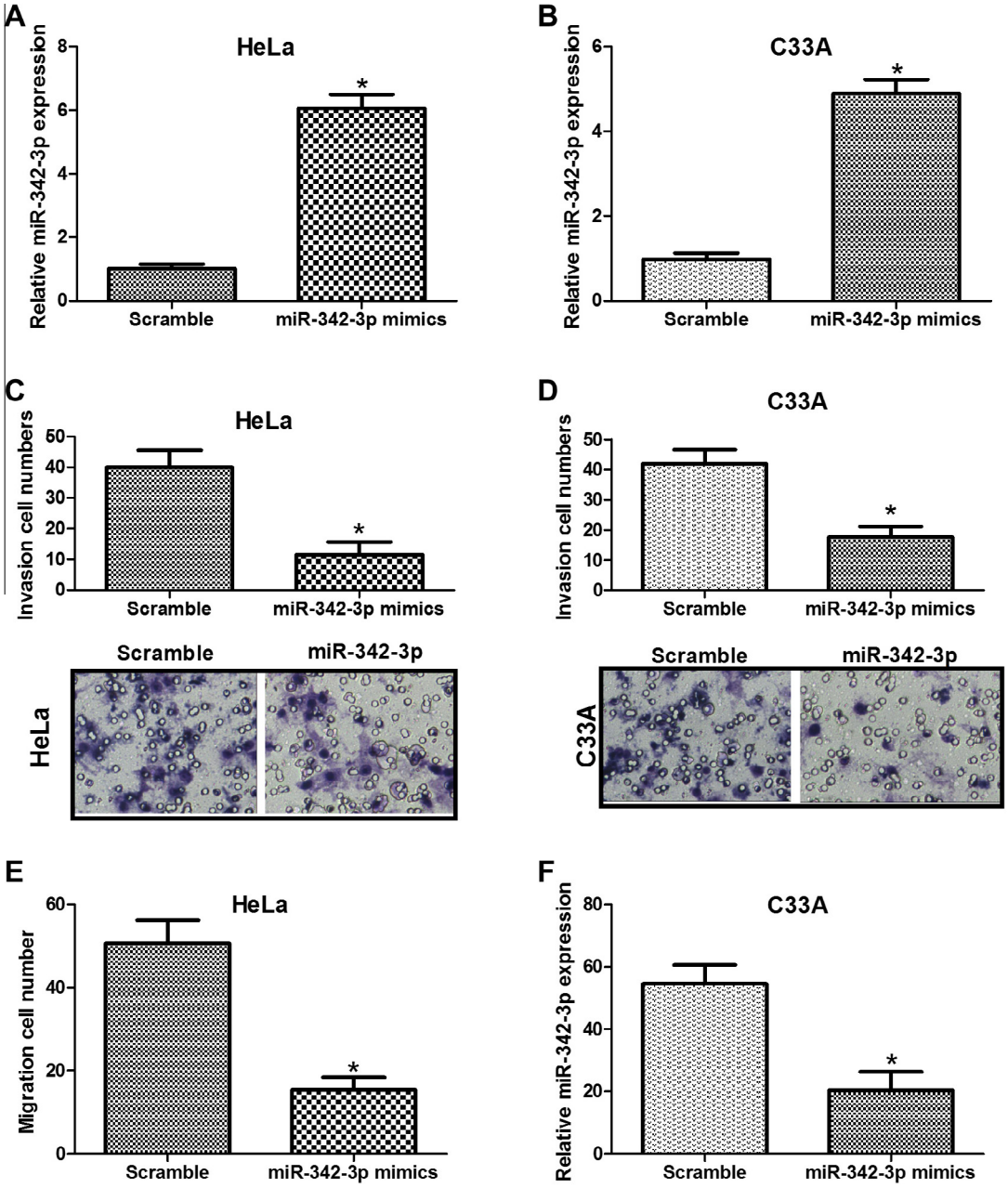

Fig. 3. miR-342-3p suppressed proliferation and growth in vitro and in vivo. Ectopic expression of miR-342-3p suppressed cell viability, proliferation and growth by MTT (A and B) and colony formation assays (C and D) in HeLa and C33A cells. Xenograft experiments were performed to confirm the effects of miR-342-3p on tumor growth in vivo. The experiments were performed in triplicate. Each group (5 mice) was injected with HeLa cells overexpressing miR-342-3p mimics or scramble. $^{*}P < 0.05$. The experiments were performed in triplicate.

sion of miR-342-3p significantly inhibited cell viability at 12 h, 24 h and 48 h after transfection and growth 10 days after seeding compared with the scramble group cells in the HeLa and C33A cell lines (Fig. 3A–D). Furthermore, to determine whether miR-342-3p is involved in tumorigenesis in vivo, a xenograft tumor model was used in the severe combined immunodeficiency (SCID) mice. Because miR-342-3p is down-regulated in cervical cancer cells, miR-342-3p mimics were used to generate a gain-of-function model. Human cervical HeLa cells transfected with miR-342-3p mimics or the control oligomers were injected into 5 nude mice. As a result, the volume of the tumors derived from the miR-342-3p mimic-treated HeLa cells was dramatically reduced at 8, 12, 16, and 20 days compared to the control group (Fig. 3E and F). The result is consistent with the effects of miR-342-3p on cultured cervical cancer cells in vitro, which indicates that miR-342-3p inhibits cell growth in vitro and in vivo.

In addition, we analyzed the effects of miR-342-3p on cell invasion and migration in the HeLa and C33A cell lines. Forced expression of miR-342-3p mimics suppressed invasion by more than 60% in HeLa cells and by more than 50% in C33A cells (Fig. 4C and D). Simultaneously, we evaluated the influence of miR-342-3p on cell migration. As shown in Fig. 4E and F, overexpression of miR-342-3p suppressed migration by more than 50% in the above two cell lines. These results indicated that miR-342-3p acts as a tumor suppressor in human cervical cells.

3.4. FOXM1 can rescue the phenotypes caused by miR-342-3p

It is reported that FOXM1 plays critical roles in cell growth, metastasis and invasion in various types of cancers, and it can be regulated by miRNAs. To study whether miR-342-3p functions through FOXM1, we constructed FOXM1 expression plasmids without the 3′UTR (pCMV6/FOXM1), which cannot be disrupted by miR-342-3p, to perform a rescue experiment. Western blotting analysis showed that overexpression of FOXM1 can restore the protein level decreased by miR-342-3p mimics (Fig. 5B). Furthermore, MTT and colony formation assays and invasion and migration assays were employed in HeLa and C33A cells co-transfected with miR-342-3p mimics and pCMV6/FOXM1 or their control oligomers and vectors. Restoration of FOXM1 abolished the cell viability, colony formation rate, invasion and migration reduced by miR-342-3p mimics (Fig. 5C–F). These results showed that FOXM1 is a direct function target gene of miR-342-3p and that miR-342-3p functions as a tumor suppressor through FOXM1.

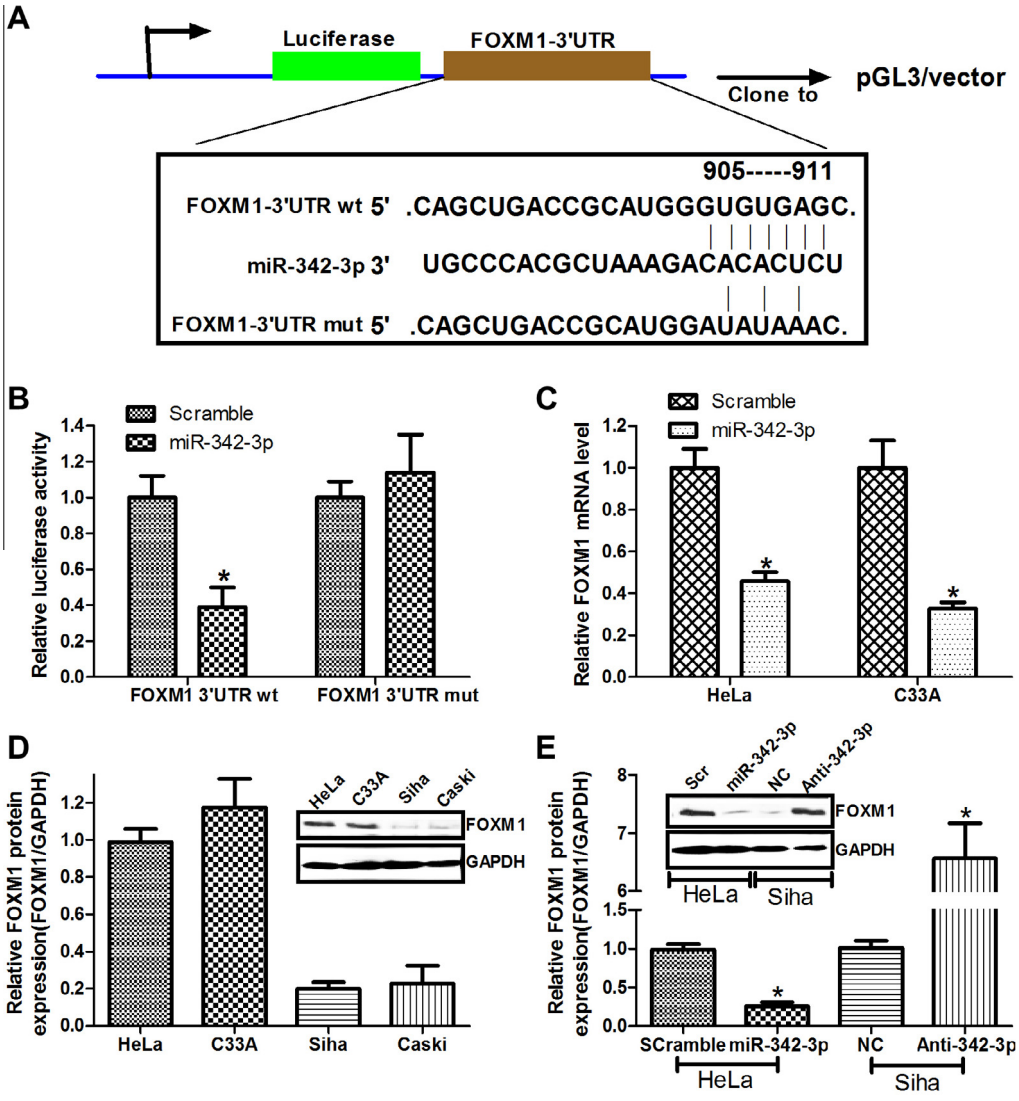

Fig. 4. miR-342-3p suppressed the invasion and migration of HeLa and C33A cells. (A and B) miR-342-3p mimics were validated effectively in HeLa and C33A cells. (C–E) Ectopic expression of miR-342-3p suppressed cell invasion and migration in HeLa and C33A cells. *$P < 0.05$. The experiments were performed in triplicate.

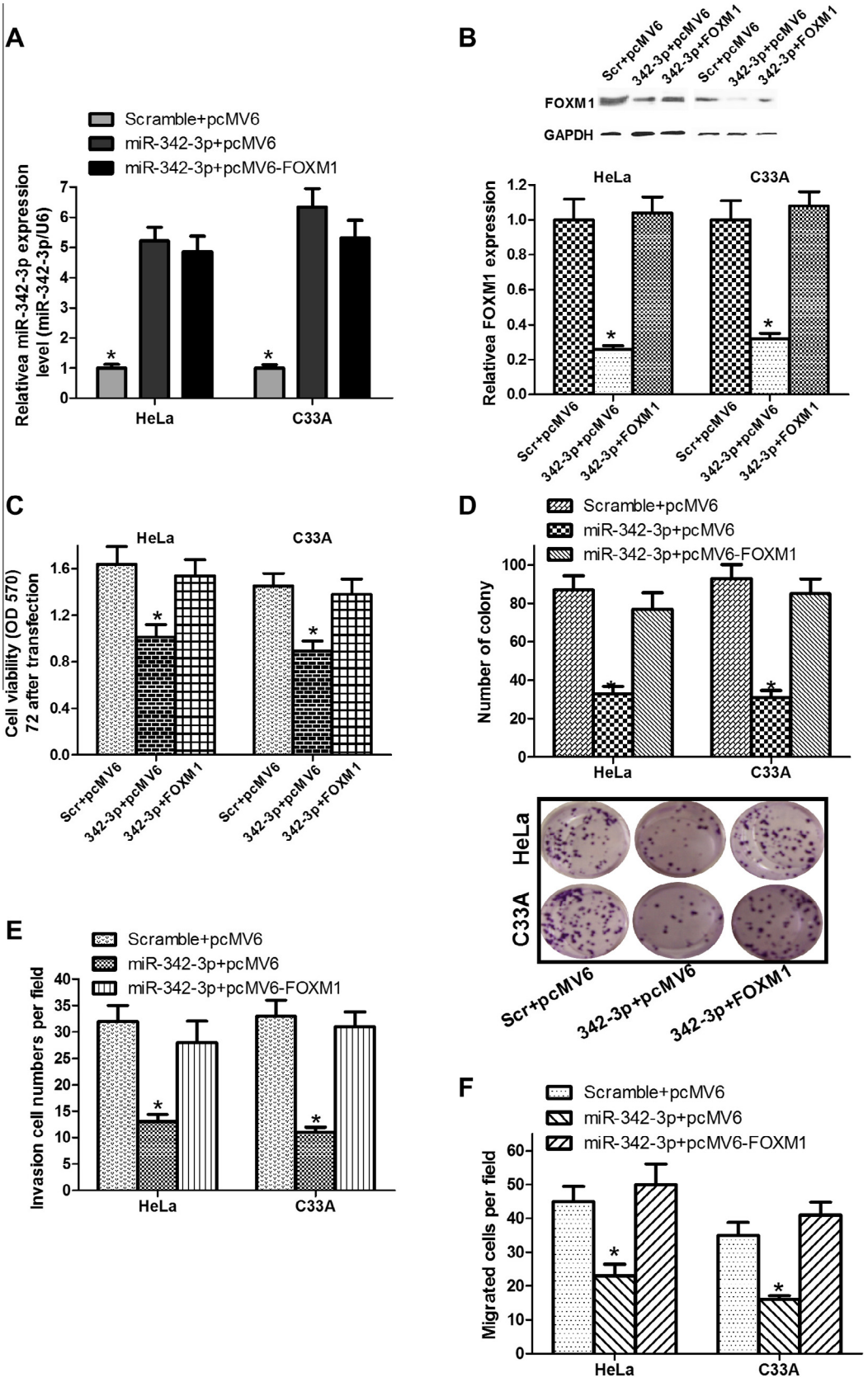

Fig. 5. FOXM1 abolished the suppression induced by miR-342-3p mimics. (A) qRT-PCR was used to analyze the expression of miR-342-3p in each group of the transfected cells. (B) Western blot assays confirmed that restoration of FOXM1 could rescue its downregulation caused by miR-342-3p mimics. (C–F) Forced expression of FOXM1 rescued the suppression effects on cell proliferation, growth, invasion and migration by MTT, colony formation assays and migration and invasion assays. $^{*}P < 0.05$. The experiments were performed in triplicate.

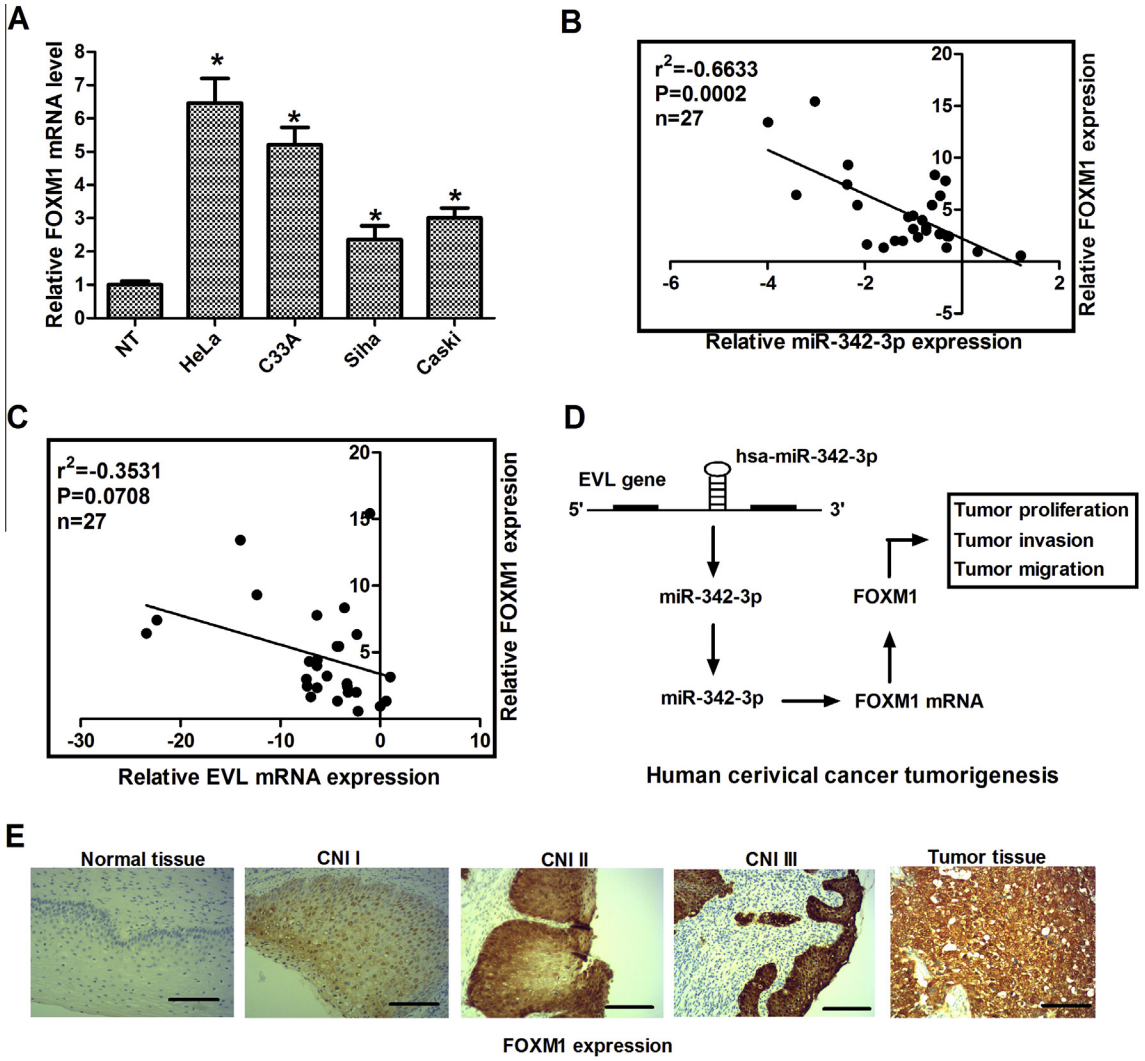

Fig. 6. miR-342-3p and its host gene, EVL, were negatively correlated with FOXM1 expression in CIN and cervical cancer tissues. (A) qRT-PCR was used to analyze the expression of FOXM1 mRNA, which is up-regulated in HeLa, C33A, Siha and Caski cell lines. (B and C) miR-342-3p and EVL were negatively correlated with FOXM1 by qRT-PCR in 27 cervical cancer tissue samples. U6 snRNA and GAPDH were used as inner controls. (D) A model of miR-342-3p function. miR-342-3p is an intronic RNA of EVL primary transcripts. (E) The expression of FOXM1 varied with the progression of CIN and was dramatically elevated in cervical cancer tissues by IHC. $^*P < 0.05$. The experiments were performed in triplicate.

3.5. FOXM1 is negatively correlated with miR-342-3p in human cervical cancer tissues

To further elucidate the association between FOXM1 and miR-342-3p, qRT-PCR was used to detect the level of FOXM1 mRNA in HeLa, C33A, Siha and Caski cell lines and 27 pairs of cervical cancer tissues and adjacent normal tissues. The levels of FOXM1 mRNA in the four cervical cell lines were relatively higher than those in normal cervical tissues, although the degrees varied from more than 2- to 7-fold (Fig. 6A). As shown in Fig. 6B, the expression of FOXM1 was upregulated in cervical cancer tissues compared with that in adjacent normal tissues, and FOXM1 and miR-342-3p had a negative correlation. Moreover, we detected the expression of the host gene EVL by qRT-PCR. The data showed that EVL was negatively correlated with FOXM1, although the correlation was not statistically significant (Fig. 6C). In addition, we performed immunohistochemistry to analyze the expression of FOXM1 protein. As shown in Fig. 6E, the levels of FOXM1 protein were higher in CIN and cervical cancer tissues than those in normal cervical tissues, and the expression of FOXM1 varied in response to human cervical malignancy (Fig. 6E). These data indicated that FOXM1 is up-regulated by miR-342-3p and thus functions as an oncogene in human cervical cancer tissues and cell lines.

4. Discussion

FOXM1 is a famous transcription factor that is involved in multiple biological processes, including cancer. The increased expression of FOXM1 has been detected in diverse cancer cell lines and cancer tissues, indicating that FOXM1 is critical for tumor cell proliferation and growth [16,17]. Elevated expression of FOXM1 is associated with tumor malignancy. A large amount of evidence suggests that FOXM1 contributes to oncogenesis [2]. The increased expression of FOXM1 promotes angiogenesis [18], invasion and metastasis [19]. In pancreatic cancer cells, downregulation of FOXM1 expression has reduced MMP-2, MMP-9, and VEGF expression, resulting in inhibition of angiogenesis, invasion, and migration [20]. Moreover, a range of studies have reported that the functions of FOXM1 in tumorigenesis can be regulated by miRNAs. For example, downregulated miR-370 contributes to proliferation and cellular senescence by up-regulating FOXM1 in acute myeloid leukemia (AML) [2]. In our study, we found that the FOXM1 mRNA

3′UTR has miR-342-3p seed sequence binding sites by using prediction tools. In the luciferase reporter assay, qRT-PCR and Western blot confirmed the direct interaction between the FOXM1 mRNA 3′UTR and miR-342-3p in HeLa and C33A human cervical cancer cell lines.

EVL belongs to the Ena/VASP (Enabled/vasodilator-stimulated phosphoprotein) family of proteins [Mena (mammalian Ena), VASP and EVL], which have a range of roles in regulating the actin cytoskeleton. Grady et al. report that expression of EVL is commonly suppressed in human colorectal cancer. And their results indicate that the mechanism of silencing is CpG island methylation upstream of EVL [14]. However, Hu et al. suggested EVL expression level was higher in breast tumors compared to normal tissues and its up-regulation was positively associated with the clinical stages of breast cancer [21]. There was also reports indicated the elevated expression levels of EVL in human glioblastoma multiforme [22]. Our study demonstrates the expression levels of the EVL mRNA were generally reduced in cervical cancer tissues compared to those in adjacent normal tissues. We speculate the different expression level of EVL may attribute to the tissues specific in different human tumors. Of course, our group will focus on the expression of EVL gene in other kind of tumors in the further study.

Accumulating evidence suggests that miRNAs play important roles in both normal cellular identity and in the pathological state, especially in tumorigenesis [23]. Among them, various miRNAs, which acts as oncogenes or tumor suppressors, have been reported to be associated with human cervical cancer. By comparing miRNA expression in normal and cervical cancer tissues, 34 upregulated miRNAs (onco-miRs) and 25 downregulated miRNAs (tumor suppressor miRs) have been found by microarray and qRT-PCR [10]. Specifically, miR-21 is overexpressed and is a negative regulator of CCL20. Overexpression of CCL20 suppresses differentiation and nodular metastasis, and thus, miR-21 binding to the CCL20 3′UTR promotes malignancy [11]. Meanwhile, many miRNAs are down-regulated in cervical cancer and act as tumor suppressors. The Cui group evaluated cell growth and apoptosis in cervical cancer cells through an MTT assay and flow cytometry and found that overexpression of miR-125b inhibited cell proliferation, induced apoptosis, and reduced tumorigenicity by targeting the phosphoinositide 3-kinase catalytic subunit delta (PIK3CD) through regulation of the PI3K/Akt/mTOR signaling pathway [24]. However, we revealed that miR-342-3p was down-regulated in human cervical cell lines and inhibited proliferation, growth, invasion and migration through targeting FOXM1, which is up-regulated in cervical intraepithelial neoplasia (CIN) tissues, cervical cancer tissues and cell lines. The degree of up-regulation of FOXM1 was directly associated with the CIN grade and cancer, and FOXM1 is negatively correlated with miR-342-3p. Moreover, miR-342 has been reported to be an intronic miRNA, and the host gene is EVL in multiple myeloma. Intronic miRNAs and host genes are regulated dependently and may contribute to the understanding of their biological roles in cancer [25]. In our research, we confirmed that the expression of miR-342-3p was identical with its host gene, EVL, which is also down-regulated in CIN as well as cervical cancer tissues and is negatively correlated with FOXM1. Furthermore, we performed rescue experiments through MTT assays, colony formation and invasion and migration assays. The data indicate that FOXM1 could rescue the suppression effects on proliferation, growth, invasion and migration induced by miR-342-3p in HeLa and C33A cells, elucidating that miR-342-3p acts as a tumor suppressor through FOXM1.

In summary, FOXM1 was predicted to be a target of miR-342-3p, an intronic RNA of EVL. The luciferase reporter assay, qRT-PCR and Western blot confirmed this direct interaction. Moreover, miR-342-3p suppressed the proliferation, growth, invasion and migration in HeLa and C33A cells. The rescue experiments confirmed that FOXM1 is a direct functional target of miR-342-3p (Fig. 6D). Finally, miR-342-3p, corresponding with its host gene, EVL, is down-regulated in CIN and cervical cancer tissues. miR-342-3p and EVL are negatively correlated with FOXM1, which is a response to the grade of CIN and cervical cancer.

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